LYSOZYME EFFECT ON SOME MEMBERS OF GENUS CLOSTRIDIUM

by

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INTRODUCTION

When first discovered, in 1922, lysozyme was evaluated mainly as a bacteriolytic agent which could have an important role in the defense mechanism of the animal body. Speculations have been made linking its restricted presence in certain tissues and secretions with the important questions of the medical bacteriologists in those days, namely, the immunogenic problems and phenomena of predilection sites.

However, the interest of the medical bacteriologists soon waned and interest in lysozyme was as quickly picked up by the microbial cytologists. Lysozyme, indeed, proved to be a tool of unrivaled neatness and gentleness for the microdissection of those microorganisms susceptible to its action. By dissolving the rigid cell wall, the building blocks of this important cell structure could be more accurately determined; and the remaining protoplast, bounded only by the protoplasmic membrane, is often stable enough for the study of subcellular units down to the molecular level.

Both the medical bacteriologists and the microbial physiologists tested lysozyme on various microorganisms, with conflicting results; however, the search was not fruitless. Conclusions have been reached on the extreme sensitivity of some organisms, the enzymic nature of lysozyme, its substrate in the cell walls, and the bonds cleaved therein.

The literature so far seen does not indicate the degree of sensitivity or resistance of the clostridia to the action of lysozyme or other cell wall degrading enzymes. This investigation was undertaken to assess the sensitivity of some members of the genus Clostridium to the enzymatic action of lysozyme.

Several criteria were used: turbidimetric, release of reducing substances, and microscopic studies.

LITERATURE REVIEW

Enzymic degradation of microbial cells was first discovered in the second decade of this century. Fleming (1922) isolated what he described as "a remarkable bacteriolytic element found in tissues and secretions". This factor, which he first isolated from nasal secretions and named lysozyme, was inhibitory to the growth of a gram-positive coccus isolated from the nasal secretions. He named the organism Micrococcus lysodeikticus, "lysis indicator".

Prevalence in Nature

Fleming (1922) found lysozyme prevalent in various tissues. The epidermal structures, the lining membranes of the respiratory tract, and connective tissue (whether fibrous, fatty, or cartilaginous) were found to contain especially high titers of lysozyme activity. Of the body fluids and effusions, tears, sputum, nasal mucus, saliva, blood serum, blood plasma, peritoneal fluid, pleural effusions, hydrocoele fluid, ovarian cyst fluid, and sebum were reported to contain lysozyme. He also reported its presence in egg white and turnips. Of all the substances examined, egg white was found to be the richest source.

Since then, lysozyme and many other "lysozyme-like ferments" from diverse sources have been isolated. Following all the reports on lytic enzymes is by no means an easy task, but perhaps a few examples may help to substantiate this statement.

Meyer et al. (1946), by turbidimetric and viscosimetric determinations on cell suspensions of M. lysodeikticus and Sarcina lutea, demonstrated the presence of mucolytic enzymes in papain and in crude extracts from several species of the tree Ficus. Ficin (Ficus extract) had the higher mucolytic activity.

Meyer and Northcote (1958) extracted similar mucolytic enzymes from the gastro-intestinal tract of the snail Helix pomatia. Since the enzymes possessed virtually no proteinase or lipase activity, but possessed $\beta(1\rightarrow 2)$, $\beta(1\rightarrow 3)$, $\beta(1\rightarrow 4)$, and $\beta(1\rightarrow 6)$ glucosidase activities, their use for cytological studies was suggested. Northcote et al. (1958) used cell wall preparations of the algus Chorella pyrenoidosa to investigate the effect of these enzymes on the microfibrillar structure of the cell walls. Upon enzymic digestion, the cell wall substrates lost 70% of their alpha-cellulose, 43% of their lipid, and 13% of their hemicellulose. Electron micrographs of the digests showed no microfibrillar structure.

Backman and Bonner (1958) obtained protoplasts from the rigid conidia and hyphae of Neurospora crassa through the action of a commercial enzyme, prepared from the snail Helix pomatia, upon the rigid conidial and hyphal walls. The enzyme was equally active on a wide range of wild types and mutant strains of N. crassa. Eddy (1958), working with cell wall preparations of the yeast Saccharomyces cerevisiae, found them to be dissolved to an appreciable degree by the snail enzyme.

McCarty (1952) used extracellular enzymes from Streptomyces albus, that were effective in lysing whole cells, to study their effect on the isolated cell wall preparation from Group A hemolytic streptococci. At least one fraction of the enzymes was found to be directed toward the carbohydrate

fraction of the cell walls; this cell wall fraction was composed primarily of N-acetylglucosamine and rhamnose -- the group-specific "C" carbohydrate. Gooder and Maxtead (1958) prepared osmotically stable protoplasts from Group A beta-hemolytic streptococci by incubating cell suspensions of known concentrations with enzymes from S. albus and with enzymes produced during bacteriophage lysis of Group C streptococci.

Salton (1955) used isolated cell walls for the preparation of cell wall agar media. The isolated cell wall suspensions of each organism were dispersed in washed agar to provide an opaque medium suitable for the isolation of cell wall decomposing microorganisms from soil. The lytic organisms were detected by the production of lysed zones in the medium. The lytic activities of isolates of actinomycetes, S. albus, two strains of Micromonospora chalceae, M. spp., Nocardia gardneri, and three strains of N. corallina were studied on media prepared from the cell walls of five gram-positive and three gram-negative bacteria and the yeast Candida pulcherina. While the Nocardia strains were found inactive against both gram-positive and gram-negative bacteria, the actinomycetes were found active against the gram-positive cell walls. Cell wall depolymerizing enzymes were investigated and their activity compared to that of lysozyme by Salton and Ghysen (1959), by Ghysen and Salton (1960), and by Ghysen (1960).

An extracellular enzyme similar to egg white lysozyme, produced during the exponential growth phase by Bacillus subtilis, was reported by Richmonds (1959a, b, and c). A "lysozyme" was even extracted from Sarcina spp. by Meyer et al. (1956b). This enzyme was found to give complete lysis of Sarcina lutea up to a dilution of 1:64. Its mode of action and properties, as reported, were similar to those of egg white lysozyme.

Properties of Lysozyme

Of all bacteriolytic enzymes, lysozyme from human and animal sources has been the most characterized. Fleming (1922) was the first to report on the properties of lysozyme. It was found to be soluble in water and normal saline, and insoluble in chloroform, ether, or toluol. It retained its potency for several weeks in aqueous solution at room temperature. It was not destroyed by desiccation, but was precipitated by protein precipitants such as acetone and picric acid. Its action needed small amounts of sodium chloride (under 0.1%) and ceased when more than 5% was present. He stated that lysozyme was very sensitive to minute traces of acid or alkali. Heating at 60°C for ten minutes did not destroy the enzyme, while it was almost inactive after heating for five minutes at 75°C. Its lytic action was found to be sluggish in the ice chest, with the reaction velocity increasing rapidly up to 60°C. Above this temperature it fell sharply, owing to the destructive action of heat on the enzyme. Lysozyme is found unable to pass through semipermeable membranes (collodion). When passed through diatomaceous earth, cotton wool, or filter paper, it was completely adsorbed by these materials until they became saturated. It could also be very quickly removed from solution by substances such as charcoal.

Wolff (1927) attempted the preparation and purification of lysozyme from egg white and the study of its chemical nature. He precipitated diluted egg white and evaporated the supernatant to a small volume. From this, lysozyme was precipitated with acetone. The aqueous solution of the precipitate was then dialyzed. The last two steps were repeated several times, and finally a white powder was obtained. It was soluble in water but insoluble in the common organic solvents. It gave a negative Molisch test and was free of

sulfur, phosphorus, and nitrogen, although the biuret reaction was weakly positive. Wolff's concepts of lysozyme were soon discarded, since it is difficult to conceive of a protein molecule which is devoid of nitrogen.

Meyer et al. (1936a) modified Wolff's method for lysozyme preparation from egg white. Egg white powder was extracted with acetic acid-alcohol mixture at 60° to 70°C for 20 to 30 minutes. The cooled extract was then filtered and the filtrate evaporated to a small volume which was then taken up in alcohol. This was allowed to stand overnight. The precipitate thus formed was washed with alcohol and taken up in slightly alkaline water which was then acidified with H_2SO_4 up to maximum precipitation. The lysozyme in the supernatant was then precipitated with flavianic acid.

The purified product of Meyer et al. (1936a) had a high activity index of 2000 to 6000 units/mg. One unit was defined as the highest dilution of lysozyme at which complete lysis of 0.5 ml of a saline suspension of Sarcina lutea, diluted to $BaSO_4$ standard number 8, took place. Their lysozyme was found to be basic in nature, being soluble in acidified aqueous media and insoluble in organic solvents. It contained about 15% nitrogen, a small amount of sulfur present as sulfhydryl, and a small amount of phosphorus. A highly purified preparation had the following composition: carbon, 48.65%; hydrogen, 6.44%; nitrogen, 15.33%; ash, 3.31%; phosphorus, 0.25%; and sulfur, 0.64%. Its molecular weight was calculated to be around 25,000. The biuret, glycoxylic acid, Greenberg phenol, and nitroprusside reactions were positive. Lysozyme solutions were not precipitated by trichloroacetic or sulfosalicylic acids and were only incompletely precipitated by tungstic acid. Perchloric acid precipitated the enzyme. Some salts of heavy metals, such as gold and silver, precipitated lysozyme with simultaneous inactivation.

While these results on the physical properties of lysozyme, contrary to Wolff's results (1927), established the protein nature of lysozyme, they were by no means in agreement with those of Fleming (1922). Lysozyme was found by them to be very stable toward heat and acid. Solutions of the enzyme in an acid medium were kept at 100°C for 45 minutes with no loss of activity. Neutral solutions of the enzyme treated in the same manner lost all activity. Heating of 100°C at pH 9 for five minutes destroyed most of the activity. Treatment with 0.01 N NaOH at room temperature for 10 minutes lowered the activity from 3000 units/mg to 80 units/mg. Lysozyme was inactivated by iodine and by cuprous oxide and could be reactivated by H_2S saturation. Peroxides and iodoacetate were also found to inactivate the enzyme. Sulfite and hydrocyanic acid were reported to partially reverse iodine and cuprous oxide inactivation. The inactivation-activation results were taken as evidence for the requirement of an intact sulfhydryl group for lysozyme action. In short, lysozyme was characterized by Meyer et al. (1936a) as a protein molecule that needs a low oxidation-reduction potential in the form of a reduced sulfur group for optimum enzymatic activity.

Abraham (1939) prepared lysozyme from egg white and obtained a pure crystalline product. His lysozyme was reported to contain 16.4% nitrogen and 3.2% sulfur. Its amino acid constituents were arginine, cystine, lysine, tyrosine, and histidine. It was shown to be a protein, soluble in water, and basic, with an acid-binding capacity of about 23 groups per molecule of enzyme.

Salton (1957) stated that current investigations had established the basic nature of lysozyme, with an isoelectric point at pH 10 to 11. It has a molecular weight of 14,700 and is characterized by the absence of sulfhydryl groups and by the high content of arginine. Lysine was found to be the N-terminal amino acid; the C-terminal amino acid is leucine (Salton, 1957).

Activity and Substrate Specificity

That lysozyme activity depended upon physical conditions such as temperature, pH, and ionic concentration has long been established (Salton, 1957).

Fleming (1922) found the activity to increase up to 60°C. Lysozyme was shown to be very resistant toward heat and acid by Meyer et al. (1936a) and by Meyer and Hahnel (1946). Warren et al. (1955) found the optimum temperature for the action of lysozyme on acetone-treated Pseudomonas aeruginosa to be 50°C, though slight activity was observed at 24°C. Peterson and Hartsell (1955) used 45°C to demonstrate sensitivity of gram-negative organisms to lysozyme by the Nakamura (1923) technique. Berger and Weiser (1957), Salton (1956), and Sohler et al. (1957) worked with lysozyme at 37°C.

The optimum pH for lysozyme action is 5.3, according to Meyer et al. (1936a and b), Meyer and Hahnel (1946), and Meyer et al. (1946). Warren et al. (1955) found lysozyme active over the relatively wide pH range of pH 5 to 9. Salton (1952) stated that he found no significant change in the rate of enzyme action over the pH range of 5.7 to 7. Berger and Weiser (1957) set the optimum pH at 6.5 to 7. All the workers in the field have stressed the importance of NaCl in small quantities for lysozyme activity. The concentrations used varied between 0.017 and 0.5 molar.

Substrate of Lysozyme

The enzymatic role of lysozyme, and its substrate in the bacterial cell wall, have only recently been known. Meyer et al. (1936b), using mild alkaline hydrolysis, obtained fractions from sarcinae which when acted upon by lysozyme liberated reducing substances. These fractions were reported to

contain nitrogen but were free from proteins and were considered to be nitrogen-containing polysaccharides of the mucoid class. No details were given of the composition or the chemical properties of these compounds.

Epstein and Chain (1940) achieved the isolation of lysozyme substrate by extracting large yields of M. lysodeikticus with antiformin or formamide. The extract of the dissoluted organisms thus obtained was then fractionated in acetone, alcohol and concentrated hydrochloric acid. The bulk of the reducing carbohydrate material came up in the acetone fraction. This fraction had a reducing power of 15%. The substrate was identified as an insoluble carbohydrate of high molecular weight which contained no protein; it gave clear solutions in 3% trichloroacetic acid, and a strong Molisch test. When acted upon by lysozyme, the substrate gave rise to soluble reducing fractions with a reducing power of 13.2%; this rose to 41% after hydrochloric acid hydrolysis. No amino groups were detected upon lysozyme action but were present after acid hydrolysis. After acid hydrolysis the material yielded 11.4% acetyl groups. These workers drew the conclusion that the products of lysozyme action were a mixture of mono- and polysaccharides, their main constituent being an N-acetylated hexosamine and a ketohexose.

Meyer et al. (1946) reported that lysozyme, in contrast to papain, had no proteolytic activity. Meyer and Hahnel (1946) prepared the crude mucopolysaccharide substrates for lysozyme from M. lysodeikticus, S. lutea, and Staphylococcus muscae. They found differences among the three mucopolysaccharide fractions in their susceptibility to lysozyme digestion. For example, the fraction from S. muscae was found to require about 30 times more lysozyme than was required for the other two. The crude M. lysodeikticus fractions were reported to contain between 5.5% and 6.5% of nitrogen and

25 to 30% of hexosamine. When these fractions were incubated with lysozyme for two hours, about 10% of the weight appeared as reducing sugar. One half of this weight was determined as acetyl glucosamine. Strange and Dark (1956) reported the presence of a substance giving the reactions of a hexosamine, but which was not glucosamine or galactosamine, in the acid hydrolysates of M. lysodeikticus.

Peterson and Hartsel (1955) proved that some of the gram-negative microorganisms were sensitive to the action of lysozyme when the technique of Nakamura (1923) was used. They stated that had Epstein and Chain (1940) used this technique, they could have found the substrate of lysozyme in appreciable amounts in this group of organisms.

Sohler et al. (1957) investigated the cell wall composition and the action of lysozyme on the cells and cell walls of the Actinomycetales. They found the cell walls of these organisms to resemble in general the gram-positive bacteria in their limited amino acid content and in that many of them were lysed by lysozyme. They confirmed other workers' results in that the action of lysozyme was found to be limited to the cell walls of susceptible microorganisms. They also stated that the substrate for lysozyme in these bacteria involved a considerable amount of hexosamine, as well as the possible presence of a phosphate ester.

However, the characterization of the bacterial cell wall structure, the molecular fractions released, and the linkages cleaved by lysozyme could be clearly visualized only in the past ten years or so, mainly through the works of Salton and co-workers. Salton (1952), working with the isolated cell walls of mechanically disrupted M. lysodeikticus, conclusively proved the depolymerizing action of this enzyme on the cell walls.

Salton (1956), working with cell walls isolated from M. lysodeikticus, S. lutea, and B. megaterium, investigated the action of lysozyme and the products of this action on the cell walls of these organisms. Ultracentrifugation, paper chromatography, electrophoresis, and sedimentation rates, among other physical and chemical tests, were used to identify the several molecular fragments in the substrates and digests of lysozyme. The major small molecular weight fragment liberated by the enzyme action was a substance giving the reactions of an acetyl amino sugar. Hydrolysis of this substance yielded a mixture of glucosamine and an unidentified substance giving the reactions of an amino sugar. The fragment was thought to be a disaccharide of glucosamine and the unknown amino sugar. Both the glucosamine and the unknown amino sugar were speculated to be present as the acetyl compounds. The material corresponding to glucosamine gave spots corresponding to glucosamine, arabinose, lyxose, and rhamnose on ninhydrin sprayed chromatograms. The unknown amino sugar, upon similar treatment, gave spots corresponding to galacturonic acid, glucosamine, glucose, arabinose, lyxose, and rhamnose. The amino acids in the undigested cell walls and in the lysozyme digests were the same. After acid hydrolysis these were found to be alanine, glutamic acid, glycine, and lysine.

Salton (1959) improved his paper chromatography technique to detect as little as 1.0 μg of N-acetylglucosamine, and applied this technique to the isolation of amino sugar compounds in partial acid hydrolysates and lysozyme digests of bacterial cell walls. In the partial acid hydrolysates N-acetyl amino sugars, free amino sugars, and free amino acids were detected. N-acetylglucosamine and a smaller amount of N-acetylmuramic acid were liberated by acid hydrolysis for six days at 40°C; in addition, an acidic compound of

N-acetylglucosamine and N-acetylmuramic acid was also detected. Hydrolysis for one and two days at 37°C yielded both N-acetylglucosamine and N-acetylmuramic acid.

Applying the same technique to the dialyzable fraction prepared from lysozyme digested walls of M. lysodeikticus, Salton (1959) found this to be composed of: (a) a compound of N-acetylglucosamine and N-acetylmuramic acid moieties, and (b) another compound which on hydrolysis yielded glucosamine, muramic acid, and alanine, glycine, glutamic acid, and lysine in the proportions of 3:1:2.4:1:1:1.4. These results together with those obtained from the partial acid hydrolysis indicated that:

(1) Repeating sequences of N-acetylglucosamine-N-acetylmuramic acid form important structural stroma in the cell walls of M. lysodeikticus.

(2) According to the above ratio of constituents, lysozyme can break the glucosidic bonds as close as three amino sugar units from the muramic acid-peptide part of the molecule.

Salton and Ghysen (1959) studied the activities of both lysozyme and the similar Streptomyces F_1 enzymes on isolated walls of M. lysodeikticus. They found that both N-acetylamino sugar compounds and N-acetylamino sugar-peptide complexes were released. Two compounds containing N-acetylmuramic acid and N-acetylglucosamine moieties, believed to be a disaccharide and a tetrasaccharide, were isolated. Both the disaccharide and the tetrasaccharide could be hydrolyzed with B-glucosidase, yielding N-acetylmuramic acid and N-acetylglucosamine. Further investigation showed that the tetrasaccharide was composed of two repeating moles of the disaccharide linked with a $\beta(1\rightarrow 4)$ linkage. The disaccharide was shown to be a compound of N-acetylglucosamine and N-acetylmuramic acid linked by a $\beta(1\rightarrow 6)$ linkage. The finding that both

lysozyme and F_1 enzymes degraded the tetrasaccharide to the disaccharide clearly indicated the $\beta(1 \rightarrow 4)$ N-acetylglucosaminidase activity of the two enzymes.

These findings were confirmed by Ghysen (1960) and by Ghysen and Salton (1960). In addition, they reported the presence, in lysozyme digests of M. lysodeikticus cell walls, several amino sugar-peptide complexes whose peptide moieties were always lysine, glutamic acid, glycine and alanine. From these and the previous results they concluded that the cell wall protein polymer is made of peptide subunits of the same type. The amino sugar polymer was believed to consist of the disaccharide and an oligosaccharide consisting of ten molecules of acetylglucosamine and ten molecules of acetylmuramic acid.

Although the nature of lysozyme action and the bonds which it cleaves were known, the problem of insensitivity of some organisms still remains to be solved. Warren et al. (1955) found P. aeruginosa cells to be more sensitive to lysozyme when treated with two volumes of acetone at 25°C for one hour. They suggested an autolytic factor within the organisms, triggered by acetone, as the cause of the increased susceptibility. Brumfit et al. (1958) obtained lysozyme-resistant variants of M. lysodeikticus by serial subculture of the organism on nutrient agar containing increasing amounts of lysozyme. It was found that this increase in resistance was due to an increase in the O-acetyl groups of lysozyme substrate. The resistance could be nullified without loss of viability by deacylation of the cell walls, accomplished by incubating the organism in 0.1 M Sorensen or glycine buffers over the pH range of 7 to 11.4. The same authors stated that in those organisms that are O-acetylated, if only every ten repeating units of the mucocomplex were deacylated, the organism could be made sensitive.

Salton (1960) stated, "But in other walls O-esters cannot account for the greater resistance of the walls to digestion with lysozyme. The possibility of different linkages between amino sugars of the backbone has been suggested. Resistance to lysozyme could also be explained by differences in the ratios of amino sugars, relatively few disaccharide units, branching points, single amino acid substituents attached to muramic acid, and a high frequency of cross linked peptides between muramic acid residues. There are many intriguing possibilities and it will be of great interest to find out the factors responsible for the resistance of the walls of an organism such as Bacillus cereus which contains such a large amount of amino sugar in the walls (30%)".

Microbial Spectrum of Lysozyme

Since its discovery, lysozyme has been tested on a vast number of microorganisms, with conflicting results. The differences in results could be due to strain differences, different growth conditions, and variations in the test conditions (Salton, 1957). Different criteria were used by the different investigators. Viability tests, respiration studies, photometric observations of turbidity changes, estimations of reducing substances liberated, and microscopic studies of lysozyme digests have been used. The last three techniques were, however, more frequently used and yielded most of the valuable information (Salton, 1960).

The three most sensitive organisms reported are M. lysodeikticus, S. lutea, and B. megaterium (Fleming, 1922; Meyer et al., 1936a and b; Epstein and Chain, 1940; Meyer and Hahnel, 1946; Salton, 1956; Berger and Weiser, 1957; Salton and Ghysen, 1959; Salton, 1959). Other gram-positive microorganisms found sensitive were streptococci (Fleming, 1922); B. subtilis,

S. griseus (Repaske, 1958); 30 species of Streptomyces (Sohler et al., 1957); and Sporosarcina ureae (Salton, 1960).

Though the gram-negative bacteria have in general a lower acetylamino sugar content in their cell walls and a higher amino acid content, and contain lipid, the susceptibility of some of them to lysozyme was found comparable to the sensitivity of some of the gram-positive microorganisms (Salton, 1960). Peterson and Hartsel (1955) found 135 organisms lysozyme-sensitive by the technique of Nakamura (1923). These belonged to the following genera, arranged in order of decreasing sensitivity: Salmonella, Brucella Shigella, Neisseria, Pseudomonas, Pasteurella, Erwinia, Escherichia, Vibrio, and Proteus. Warren et al. (1955) reported Pseudomonas aeruginosa to be sensitive to lysozyme if previously treated with acetone. Repaske (1956, 1958) successfully lysed E. coli, P. aeruginosa, P. fluorescens, Desulfovibrio desulfuricans, Flavobacterium sp., and Azotobacter vinelandii with lysozyme and versene.

Protoplast Formation

Recently it has been found that lysozyme action on susceptible microorganisms and consequent removal of the cell wall in suitable media gave rise not to lysis but to spherical osmotically sensitive structures for which the name of protoplast was coined. These protoplasts were characterized as follows (Mahler and Frazer, 1956; McQuillen, 1956; Weibul, 1956).

(1) They are derived by structural alteration of the cells in such a way as to render them incapable of producing colonies under the usual plating conditions.

(2) They still possess much of the organization of the cell, being definite morphological entities as distinguished microscopically; and their suspensions retain much of the turbidity of the original cell suspensions.

(3) They are stable in suitable hypertonic solutions but instantly lyse in hypotonic solutions.

(4) They retain much of the synthetic capabilities of the original intact cells.

(5) They can sporulate if the cells were committed before treatment.

(6) They can be infected with bacteriophage if it adhered before treatment.

(7) The change to protoplasts is irreversible.

Lysozyme protoplasts have been formed from M. lysodeikticus by Salton (1952), Grula and Hartsel (1954), and by Mitchell and Moyle (1956). They were also formed from B. megaterium (Weibul, 1953a and b).

Weibul (1953a) found the protoplasts from B. megaterium to be stable if kept semianaerobically in 0.1 to 0.2 molar sucrose or 7.5% polyethylene glycol. Other efficient stabilizers for protoplasts are 0.3 M glucose and 1.5 M raffinose, melibiose, or trehalose (Weibul, 1956). Mitchell and Moyle (1956) stated that protoplasts of M. lysodeikticus and S. lutea could be stabilized in 1.5 molal solutions of the following solutes: NaCl, KCl, NH_4Cl_2, sodium acetate, potassium acetate, NaBr, K_2SO_4, (KH_2PO_4 and K_2HPO_4), sodium glutamate, lysine hydrochloride, glucose, and sucrose. In 1.5 M glycerol the protoplasts lysed almost instantaneously. S. aureus protoplasts were reported to be very stable in 1.5 molal NaCl but lysed quickly in a similar concentration of NaCNS. The fact that a 1.5 molal solution of glycerol failed to

stabilize the protoplasts showed the stability with the other solutions was due to the substitution of an osmotic pressure at the outer surface of the protoplast membrane for the hydrostatic pressure exerted by the cell wall.

"Protoplasts" were produced by agents which interfered with cell wall synthesis. Lederberg and Clair (1958) produced "protoplasts" from E. coli grown in the presence of penicillin or deprived of diaminopimelic acid. The "protoplasts" were found to lyse in diluted media but could be maintained in protective media containing M/3 sucrose plus M/100 Mg^{++}. In penicillin sucrose broth the "protoplasts" continued to enlarge but did not proliferate. In the absence of penicillin they reverted to form normal E. coli cells.

MATERIALS AND METHODS

Cultural

The following cultures of Clostridium species and strains were collected from various sources and given the designations "F", "L", and "V" according to the source from which they were obtained:

Species	Strain	Source
Cl. tetani	F	Prof. V. D. Foltz
Cl. perfringens	F	Prof. V. D. Foltz
Cl. perfringens	V	Dr. Theodore Vera
Cl. perfringens	L	Dr. T. H. Lord
Cl. sporogenes	F	Prof. V. D. Foltz
Cl. sporogenes	V	Dr. Theodore Vera
Cl. sporogenes	L	Dr. T. H. Lord
Cl. septicum	F	Prof. V. D. Foltz
Cl. septicum	V	Dr. Theodore Vera
Cl. hemolyticum	L	Dr. T. H. Lord
Cl. hastiforme	V	Dr. Theodore Vera
Cl. multifermentans	V	Dr. Theodore Vera
Cl. multifermentans	L	Dr. T. H. Lord
Cl. aerofoetidum	L	Dr. T. H. Lord
Cl. tertium	L	Dr. T. H. Lord
Cl. butyricum	L	Dr. T. H. Lord
Cl. lentoputrescens	L	Dr. T. H. Lord

These cultures were collected and maintained in Difco egg meat medium throughout this work. About 1.5 grams of Difco dehydrated egg meat medium were dispensed in each screw-capped tube (150x20) and the medium then rehydrated with 15 mls of distilled water. After shaking for 10 to 15 minutes to soak the meat balls, the tubes were autoclaved at 15 pounds pressure for fifteen minutes (Difco Manual, 1953). The cultures were transferred every two to three weeks to insure viability.

To obtain enough cells for lysozyme action tests, a fluid basal medium with the following composition was prepared:

Bacto tryptose	10 grams
Difco yeast extract	3 grams
Glucose	10 grams
Water	to 1000 mls

The ingredients were dissolved by steaming for 20 minutes. The pH was standardized to pH 7.2 to 7.4 and the medium dispensed in 12 ml amounts in screw-capped tubes. These were sterilized at 15 pounds pressure for fifteen minutes. The redox reagents sodium thioglycollate, sodium thiosulfate, and sodium sulfite were prepared in 10% solutions and sterilized separately. Addition of these redox reagents in 0.1% concentrations of the basal medium was carried out aseptically, immediately before inoculation of the test organisms. To choose the basal medium-redox combination that would yield the best results, 11 organisms from the above list were inoculated into each of the three redox media plus a basal medium control. These were incubated overnight at 37°C with the caps tight. Sodium thioglycollate was found, by visually comparing the turbidities of the cultures, to yield the most luxuriant growth of all the organisms tested. The test medium was therefore made by preparing the basal medium as described above, except for dispensing in 100 ml amounts in

6-ounce prescription bottles with screw caps. Sodium thioglycollate was prepared in 10% concentration, sterilized separately, and added in 0.1% concentration to each bottle immediately before inoculation. The basal medium and the redox reagent were either used fresh, or, if old, steamed for 15 minutes and cooled before inoculation. This medium will be referred to as thioglycollate broth.

Preparation of Resting Cell Suspensions. The following reagents were used in the preparation of the resting cell suspensions: (1) thioglycollate broth as described under "Cultural", and (2) mineral salts solution prepared as follows:

K_2HPO_4, anhydrous	14.4 grams
KH_2PO_4	5.6 grams
$MgSO_4 \cdot 7H_2O$	1.6 grams
NaCl	1.6 grams
$CuCl_2$, anhydrous	.16 gram
$FeCl_3$	.2 ml
Microelements	.8 ml of stock solution
Water	to 4 liters

(The microelements stock solution was made up of .5% of each of the following: molybdic oxide, manganese sulfate, copper sulfate, cobalt acetate, zinc chloride, potassium iodide, and borax.)

To one liter of the above solution, the following were added: distilled water, three liters; K_2HPO_4, 10 grams; KH_2PO_4, 9 grams; and $(NH_4)_2SO4$, 8 grams (Harris, 1961).

Lysozyme Solution. Armour Laboratories crystalline lysozyme was reconstituted by dissolving in distilled water at the concentration of 1 mg/ml.

The solution was clear and homogeneous and was usable for weeks if kept in the refrigerator at 4°C. This solution will be referred to as "lysozyme" throughout this work.

Experimental

The sensitivity of resting cell suspensions suspended in mineral salts solution was tested (1) turbidimetrically; (2) for reducing sugar release; and (3) for lysis or changes in morphology.

Turbidimetric Determinations. The changes in turbidity resulting from lysozyme action on the cells were assessed by measuring the optical density changes of the suspension in a spectrophotometer. A Bausch & Lomb spectrophotometer type 33-29-40 was used throughout these experiments.

Resting cell suspensions of each of the test organisms were diluted to give an optical density in the middle of the scale. For testing any one organism, 3 ml amounts of the resting cell suspensions were pipetted into two spectrophotometer tubes and the optical density of the two tubes read in the spectrophotometer at a wavelength of 600 mμ. To one tube, 1 ml of lysozyme was added; to the second tube, 1 ml of mineral salts solution was added as a control. Immediately after mixing, the optical density of each tube was again measured. The two tubes were then set on the bench and their optical density readings taken every 10 minutes until 30 minutes after the first readings; thereafter, readings were taken every 30 minutes until two hours after the first reading. The suspensions were always shaken before the readings were taken, to insure thorough mixing and to avoid erroneous results due to auto-agglutination of cells or to settling of the suspensions.

Reducing Sugar Release. Test reagents necessary for carrying out the sugar reduction tests by the method of Folin and Malmros (1929) were prepared as follows:

(1) A .4% solution of potassium ferricyanide was prepared by dissolving 0.4 gram in 100 mls of distilled water.

(2) Carbonate-cyanide solution was prepared by dissolving 8 grams of anhydrous sodium carbonate in 50 mls of distilled water and adding 15 mls of freshly-prepared 1% sodium cyanide. After thorough mixing, the solution was diluted to 500 mls with distilled water.

(3) Ferric iron solution: Twenty grams of gum ghatti were soaked in 1 liter of water for several hours, the gum being suspended in cheesecloth in the water. A mixture of 5 grams of ferric sulfate, 75 mls of 85% phosphoric acid, and 100 mls of distilled water was then added. After thorough mixing, a 1% solution of potassium permanganate was slowly added until the pink color of the permanganate just appeared. (This was necessary to destroy reducing materials already present in the gum.) The solution was then allowed to stand for two days before use.

To prepare a glucose standard, glucose solutions containing 5 µg/ml and 20 µg/ml were first prepared. The appropriate amounts of glucose, as shown in the following table, were pipetted into pyrex test tubes and the sugar reduction test of Folin and Malmros (1929) run on them. Concentration range chosen was from 5 µg to 80 µg. Any sample of less than four mls was made up to this amount with distilled water.

Glucose conc./ml	Mls tested	Amount of glucose tested
5 µg/ml	1 ml	5 µg
5 µg/ml	2 mls	10 µg
5 µg/ml	3 mls	15 µg
5 µg/ml	4 mls	20 µg
20 µg/ml	1.25 mls	25 µg
20 µg/ml	1.5 mls	30 µg
20 µg/ml	1.75 mls	35 µg
20 µg/ml	2 mls	40 µg
20 µg/ml	2.25 mls	45 µg
20 µg/ml	2.5 mls	50 µg
20 µg/ml	2.75 mls	55 µg
20 µg/ml	3 mls	60 µg
20 µg/ml	3.25 mls	65 µg
20 µg/ml	3.5 mls	70 µg
20 µg/ml	3.75 mls	75 µg
20 µg/ml	4 mls	80 µg

To each 4-ml sugar aliquot, two mls of the potassium ferricyanide solution and one ml of carbonate-cyanide solution were added. The sample was mixed and heated for eight minutes in a boiling water bath, then cooled for two minutes. Five mls of ferric iron solution were added. A strong blue color and sediment developed. The volume was then made up to 25 mls with distilled water in graduated tubes, mixed thoroughly, and the solution's optical density measured in the spectrophotometer at a wavelength of 600 mµ. Results obtained were treated graphically by plotting the optical density values against the glucose concentrations used.

A resting cell suspension of Cl. sporogenes F was diluted and the turbidity of the dilute suspension determined by measuring the percentage transmission in the spectrophotometer at 600 mµ. A Petroff-Hausser count was then carried out on this suspension.

The total count for this organism corresponding to percentage transmission of 71.5% was found to be 1.9×10^7/ml. This was used as the standard for the determination of total counts for the other organisms tested. Aliquots

from each resting cell suspension tested were diluted with known amounts of mineral salts solution to give a percentage transmission reading of 71.5%, and the total count calculated by multiplying the standard count by the dilution factor used.

After determining the total count/ml, three mls of resting cell suspension of each test organism were pipetted into each of two tubes 200 x 12 mm. To one tube was added 1 ml of lysozyme, and to the second tube 1 ml of mineral salts solution was added. To a third tube 1 ml of lysozyme was added to 3 mls of mineral salts solution. The contents of the three tubes were thoroughly mixed and allowed to stand on the bench for two hours. Next, the tubes were centrifuged at 5000 rpm for 15 minutes. One ml from the supernatant was then pipetted into a separate test tube. The sugar reduction test on these aliquots was run exactly as described for the standard glucose solution. Comparing the optical density value obtained in each case with that of the standard glucose, the glucose equivalents of reducing material liberated could be calculated by inspection of the standard glucose graph (Fig. 1).

Lysozyme was found to possess an appreciable reducing value, as did the control tubes of resting cell suspensions. The amount of reducing material was therefore calculated as follows:

G μg = the amount of reducing material released by lysozyme from a resting cell suspension.

T = the total reducing material from 1 ml of supernatant of lysozyme-digested resting cells.

t = the reducing material from 1 ml of supernatant of resting cell suspension.

E = the reducing material liberated from 1 ml of lysozyme.

$G = T - (t + E)$.

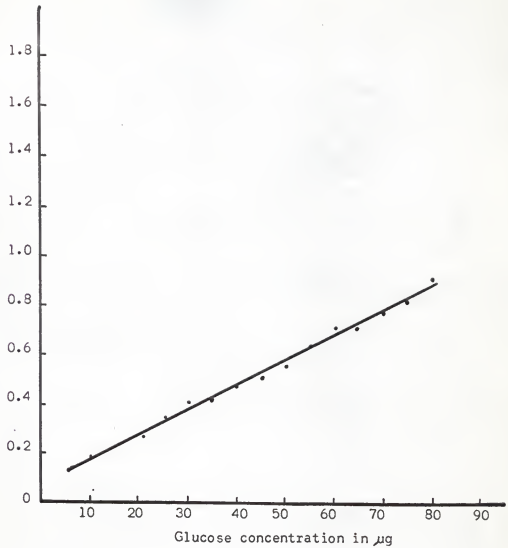

Fig. 1. Glucose standard: optical density at 600 mμ plotted against glucose concentrations in ug.

Examination for Lysis or Change of Morphology. In the beginning, wet smears from the experiments performed for the turbidimetric determinations were examined, after the two hours of incubation, in the phase contrast microscope using a 450X magnification and green filters. This same process was repeated using 20% sucrose as the suspending medium.

Later, a set of experiments was performed interacting each resting cell suspension of the test organism and lysozyme, as described for the turbidimetric determinations. After two hours of incubation at room temperature, 0.1 ml of each resting cell suspension was pipetted into 3 mls of each of the following electrolytes:

(1) 3 molal, 1.5 molal, and 1 molal sodium chloride

(2) 3 molal, 1.5 molal, and 1 molal glycerol

(3) 3 molal, 1.5 molal, and 1 molal sodium thiocyanide

(4) EDTA solution of the composition: Disodium ethylenediamine tetraacetate in 2000 μg/ml solutions in distilled water (Frazer and Mahler, 1957).

The tubes were then mixed and allowed to stand on the bench for half an hour. The suspensions were centrifuged at 5000 rpm for 15 minutes and the supernatant was discarded. From the sediment, smears were made, dried, fixed, and stained with Gram's stain. These were examined under the light microscope at 1000X magnification, using the oil immersion lens.

EXPERIMENTAL FINDINGS

Turbidimetric Determinations

Turbidimetric studies for the investigation of lysozyme action on bacterial cell walls have been used by many workers in this field. Salton

(1953) studied turbidimetrically the effect of lysozyme on E. coli, P. fluorescens, Spirillum serpens, B. megaterium, M. lysodeikticus, and S. fecalus. His studies were conducted on untreated bacteria suspended in 0.1 M phosphate buffer and on similarly suspended bacteria treated at 100°C for five minutes. In all these experiments a lysozyme final concentration of 1 mg/ml was used. With the untreated M. lysodeikticus a marked decrease in turbidity was observed. With heat-killed E. coli and P. fluorescens an optical density increase of about 20% to 30% was observed. This increase was attributed to the fact that lysozyme as a basic molecule would adhere to the negatively charged bacterial cells. A similar increase in turbidity was observed with heated suspensions of B. megaterium, but because the electron micrograms of the digests showed the organisms to have been reduced to many short rods with square ends, it was concluded that the optical density increase in this case was due to the actual removal of the cell wall by the enzyme. Turbidity changes resulting from lysozyme action on the isolated cell walls of M. lysodeikticus, S. lutea, and B. megaterium were followed photometrically and expressed as percent decrease in the optical density (Salton, 1956).

Peterson and Hartsell (1955), in their well-controlled experiments on the effect of lysozyme on gram-negative organisms, expressed their results as percent of clearing. They demonstrated that there were differences in clearing among the different genera and among strains of the same species. The salmonellae showed an appreciable clearing of between 32% and 78%, while the Escherichia strains demonstrated much less clearing, between 18% and 38%. Salmonella typhosa strain #24 showed 72% clearing; strain #55 showed 48% clearing; and strain #25 showed 38% clearing.

In the present investigation, increases in the optical densities were observed for all the organisms tested. These results, expressed in percent increase in optical density versus time, are shown in Table 1 and Figs. 2, 3, 4, and 5. All the controls showed the expected decrease in optical density resulting from the dilution of the suspensions with mineral salts solution. However, after this initial immediate decrease, only slight variations in the optical density readings took place (Table 1).

The test suspensions, except for Cl. tetani F and Cl. lentoputrescens L, showed an immediate marked increase in their optical densities. The amount of this immediate increase varied from one organism to another. There were species differences as well as strain differences. Cl. sporogenes V showed an initial increase in optical density of 6% while Cl. multifermentans V showed an increase of 16%, although the two suspensions had nearly the same optical density reading before the addition of lysozyme, being 0.225 and 0.215, respectively.

Cl. multifermentans V showed an initial optical density increase of 16% while Cl. multifermentans L showed an 11% increase. Cl. tetani F and Cl. lentoputrescens L showed an immediate decrease in optical density equal to that of their respective controls. The optical density readings for Cl. tetani F started to rise at the first ten minutes of incubation and showed consecutive rises at 20, 30, and 120 minutes of incubation. Cl. lentoputrescens L suspensions showed a more marked rise at the end of the first ten minutes of incubation.

Table 1. Percentage optical density changes of lysozyme-treated resting cell suspensions, and controls.

Test organisms and controls	Starting optical density	Percentage increase or decrease in optical density					
		Immediate	10 min.	20 min.	30 min.	60 min.	120 min.
Cl. tetani F	0.21	-3.5	+4	+5.5	+8	+9	+12
Cl. tetani F control	0.205	-9.5	-8.5	-8.5	-8	-8	-8
Cl. perfringens F	0.21	+14	+21	+21.5	+21.5	+21.5	+21.5
Cl. perfringens F control	0.205	-6	-6	-5.5	-5.5	-5.5	-5.5
Cl. perfringens V	0.31	+21	+37	+39	+30	+30	+20
Cl. perfringens V control	0.30	-8	-6.5	-6.5	-6.5	-6.5	-6.5
Cl. perfringens L	0.268	+13.2	+16.7	+16.7	+16.7	+16.7	+17.7
Cl. perfringens L control	0.268	-7.8	-7.3	-7.3	-8	-8	-8.8
Cl. sporogenes F	0.24	+2	+20	+20	+22	+24	+26
Cl. sporogenes F control	0.24	-5	-4.9	-4.9	-4.9	-4.9	-5
Cl. sporogenes V	0.225	+6	+7.5	+4.5	+5	+5	+5
Cl. sporogenes V control	0.225	-8	-7.5	-7.5	-7.5	-7.5	-7.5
Cl. sporogenes L	0.275	+13.5	+22.5	+22.5	+22.5	+22.5	+22.5
Cl. sporogenes L control	0.275	-7	-7.5	-7.5	-7.5	-7.5	-7.5
Cl. septicum F	0.235	+6	+9	+9	+9	+9	+9
Cl. septicum F control	0.235	-6	-5.5	-5	-4	-4	-5.5
Cl. septicum V	0.215	+8.5	+8.5	+8.5	+8.5	+8.5	+8.5
Cl. septicum V control	0.215	-6	-6.5	-6.5	-6.5	-6.5	-6.5
Cl. hemolyticum L	0.02	+6.5	+7.8	+10	+16	+16	+16
Cl. hemolyticum L control	0.02	-1	-1	-1	+1	-1	-1

Table 1 (concl.).

Test organisms and controls	Starting optical density	Percentage increase or decrease in optical density					
		Immediate	10 min.	20 min.	30 min.	60 min.	120 min.
Cl. hastiforme V	0.145	+28	+28	+29	+29.5	+29.5	+29.5
Cl. hastiforme V control	0.145	-4.5	-4.5	-3.7	-4.6	-4.6	-5.4
Cl. multifermentans V	0.215	+16	+18.5	+16.5	+16.5	+16.5	+16
Cl. multifermentans V control	0.215	-8.5	-8	-8	-8	-8	-8
Cl. multifermentans L	0.24	+11	+19	+19	+19	+19	+19
Cl. multifermentans L control	0.24	-6.5	-6	-5.5	-6.1	-7	-7
Cl. aerofoetidum L	0.239	+10.1	+19.1	+19.6	+19.6	+19.6	+19.6
Cl. aerofoetidum L control	0.239	-5.4	-5.9	-5.4	-5.4	-6.4	-6.4
Cl. tertium L	0.139	+9.1	+16.6	+18.6	+19.6	+19.1	+19.1
Cl. tertium L control	0.139	-2.4	-2.4	-2.4	-2.4	-2.4	-2.4
Cl. butyricum L	0.14	+6.5	+13.5	+13.9	+13.9	+13.2	+14.5
Cl. butyricum L control	0.14	-4	-4	-4	-3.5	-4.2	-4
Cl. lentoputrescens L	0.165	-2.5	+11	+13.5	+14.5	+14.5	+14
Cl. lentoputrescens L control	0.168	-2.8	-2.8	-2.8	-2.8	-2.9	-2.9

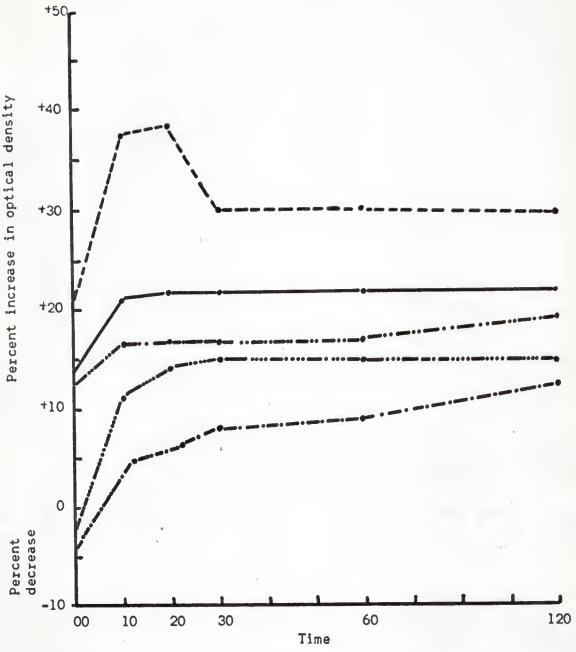

Fig. 2. Percent increase or decrease in optical density against time in minutes.

Cl. perfringens V
Cl. perfringens F
Cl. lentoputrescens L
Cl. perfringens L
Cl. tetani F

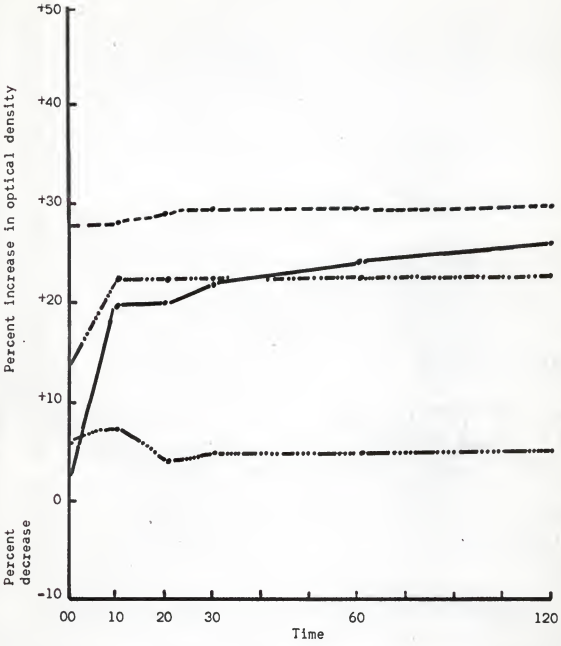

Fig. 3. Percent increase or decrease in optical density plotted against time in minutes.

Cl. hastiformi V
Cl. sporogenes F
Cl. sporogenes L
Cl. sporogenes V

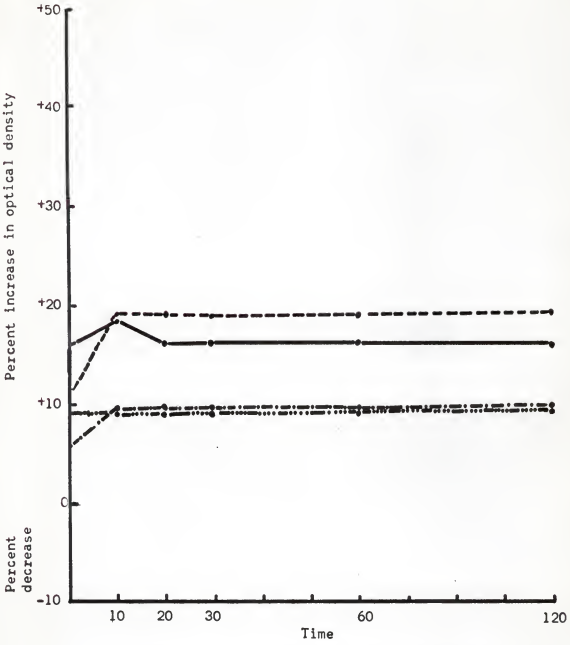

Fig. 4. Percent increase or decrease in optical density plotted against time in minutes.

Cl. multifermentans L
Cl. multifermentans V
Cl. septicum F
Cl. septicum V

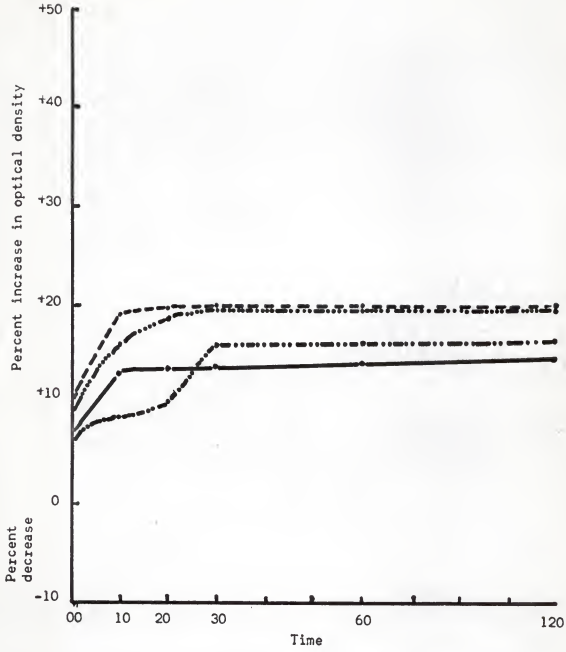

Fig. 5. Percent increase or decrease in optical density plotted against time in minutes.

Cl. aerofoetidum L
Cl. butyricum L
Cl. hemolyticum L
Cl. tertium L

Reducing Sugar Release

Release of reducing substances from lysozyme-digested cell or cell wall suspensions have been reported by most of the investigators since the first report of Epstein and Chain (1940); Meyer et al. (1946); Sohler et al. (1957); Salton (1956, 1959). Their results were expressed as percent reducing sugar released from a given weight of dried organisms or cell walls.

In this work, the results of reducing sugar released were arrived at in the manner described under Experimental. These, together with the counts per milliliter for each organism, were found to be as follows:

Organism	Strain	Total Count/ml	Reducing material released (μg/ml)
Cl. tetani	F	9.5×10^6	none
Cl. perfringens	F	5.7×10^8	none
Cl. perfringens	V	4.75×10^8	none
Cl. perfringens	L	5.7×10^8	none
Cl. sporogenes	F	1.9×10^8	none
Cl. sporogenes	V	9.5×10^7	none
Cl. sporogenes	L	3.8×10^7	none
Cl. septicum	F	3.17×10^8	none
Cl. septicum	V	1.14×10^8	none
Cl. hemolyticum	L	9.5×10^6	none
Cl. hastiforme	V	1.9×10^7	none
Cl. multifermentans	V	7.6×10^7	none
Cl. multifermentans	L	1.007×10^9	20
Cl. aerofoetidum	L	1.14×10^9	none
Cl. tertium	L	7.6×10^8	19.5
Cl. butyricum	L	5.13×10^8	none
Cl. lentoputrescens	L	5.7×10^8	11.5

The only organisms in which there was a net increase in reducing power were Cl. multifermentans L, Cl. tertium L, and Cl. lentoputrescens L. For these organisms, the net reducing sugar release as compared to the glucose standard was expressed in μg/ml of suspension. The amount of reducing material released seemed to conform to the total counts/ml: the higher the number of cells per milliliter, the higher was the yield of reducing material.

Lysis or Change in Morphology

No lysis was observed in either the original experiments or the subsequent experiments using sodium chloride, sodium thiocyanide, glycerol, and EDTA. However, the following organisms were observed to become distorted, when gram stained and observed under 1000X oil immersion lens, after the half hour incubation in sodium chloride, sodium thiocyanide, or glycerol: Cl. hemolyticum L, Cl. multifermentans L, Cl. butyricum L, and Cl. lentoputrescens L. The organisms appeared slightly distorted, with flattened ends.

No protoplasts were observed in the original experiments when wet smears from the test suspensions were examined in the phase contrast microscope, or when the same cells were suspended in 20% sucrose. Neither were protoplasts or their ghosts detected when the test suspensions were transferred to the chelating agent EDTA or to the hypertonic protective solutions of sodium chloride and sodium thiocyanide.

DISCUSSION

Optical density increases of bacterial suspensions could be an outcome of the growth and resultant increase of the given population. In fact, this

method is commonly employed for determining the rate and extent of growth in bacterial cultures. Optical density increases were reported to have occurred with thymine-deficient E. coli and S. fecalis grown in media deficient in theonine, leucine, isoleucine, valine, histidine, methionine, or arginine. In the case of E. coli, the cells were reported to have increased in length and diameter, with the doubling of their ribonucleic acid content. This was explained on the basis of the occurrence of continuous cytoplasmic growth without nuclear division (for which thymine is required). After valine depletion from the media, S. fecalis showed an optical density increase of 50% (McQuillen, 1958).

Salton (1953) obtained immediate optical density increases of 20% to 30% when lysozyme in the concentration of 1 mg/ml was added to heat-killed suspensions of E. coli and P. fluorescens. He obtained a similar optical density increase with heat-killed B. megaterium. Since B. megaterium, in contrast to the gram-negative organisms, was shown in the electron microscope to have been reduced to many, small bacilli with square ends, the optical density increases in the two cases where explained differently. It was stated that when heat-killed bacteria were treated with high concentrations of lysozyme, the optical density increase would be due to a physical interaction between the negatively charged bacterial surface and the positively charged basic enzyme molecules. This observation was confirmed by the similar rise in the optical density of heat-treated bacterial suspensions when high concentrations of the basic protamine salmine were added. The increase in optical density of B. megaterium was attributed to the action of lysozyme on the organisms.

The optical density increases obtained in this work could not be explained by either population increase or metabolic disturbances, because of the inapplicability of these phenomena to resting cell suspensions. However, though the cells had not been previously heat treated, the possibility that the optical density increases observed could be due to the basic properties of lysozyme was difficult to dismiss. This assumption appeared more likely with the organisms in which an immediate optical density increase occurred with little or no change throughout the two-hour period of incubation, namely Cl. sporogenes F, Cl. septicum V, and Cl. multifermentans V. On the other hand, the fact that the lysozyme concentration used was only 250 ug/ml would make this possibility appear rather remote.

Considering the results of reducing sugar release, only Cl. multifermentans L, Cl. lentoputrescens L, and Cl. tertium L gave reducing values (20 μg/ml, 19.5 μg/ml, and 11.5 μg/ml, respectively). These results are an additional indication that in these organisms, lysozyme must have depolymerized in whole or in part the mucopolysaccharide content of the cell walls. There could have been no other source of reducing sugar release, since the reducing values of lysozyme and of the resting cell suspension were subtracted from the total reducing value. The reducing values obtained could therefore represent only reducing material, probably N-acetylglucosamine and its disaccharide, liberated by the action of lysozyme on the cell walls.

The abortive attempts to engender lysis of the cells or alternatively to observe the release of protoplasts from any of the organisms tested could not under these circumstances be taken as a criterion for lysozyme inactivity against these organisms. Fleming (1922) made the observation that while

lysozyme was lytic toward some organisms, it killed others without causing lysis. Epstein and Chain (1940) found B. subtilis, B. anthracis, and an unidentified air organism to have been made nonviable by lysozyme action without showing any sign of lysis. They also showed that mucopolysaccharides were released by the action of the enzyme on these organisms. Meyer and Hahnel (1946) stated that lysozyme lysis was probably associated with bacterial autolytic enzymes. Peterson and Hartsell (1955) made the statement that lysozyme lysis appeared to be a complex phenomenon involving diffusion, substrate degradation, solubility, and the presence of autolytic enzymes.

That the organisms used in this work did not show lysis was attributed to the probability that only a small fraction of the mucopolysaccharide backbone was hydrolyzed by lysozyme. This assumption would be particularly applicable to those organisms which yielded reducing material.

The microscopic picture of the gram stained smears in the various salt concentrations was suggestive. Of the three organisms which showed reducing sugar release, only Cl. tertium L appeared normal. Though the gram stain cannot be considered a cytological stain for cell wall demonstration, the appearance of distorted cells in the test suspensions of Cl. hemolyticum L, Cl. butyricum L, Cl. multifermentans L, and Cl. lentoputrescens L could not be overlooked. The observation that all the test cells appeared normal in the phase contrast microscope led to the conclusion that the hypertonic salt solutions, together with the heat used for drying and fixing of the smears, augmented whatever impairment lysozyme had on the cell walls of these organisms.

SUMMARY OF RESULTS AND CONCLUSIONS

The effects of lysozyme on some of the clostridia were assessed turbidimetrically, for reducing sugar release, and for lysis and protoplast formation.

All the organisms tested showed an ultimate, significant increase in turbidity. The possible causes underlying these increases, and their significance, were discussed. The possibility that these increases were due to the basic properties of lysozyme was alluded to but was found incompatible with the lack of previous heat treatment for the organisms, and with the low concentration of lysozyme used.

Only three organisms showed reducing values: Cl. multifermentans L, Cl. lentoputrescens L, and Cl. tertium L. The reducing values obtained were observed to increase with the total count. From these findings it was concluded that lysozyme must have depolymerized, at least in part, the mucopolysaccharide content of the cell walls in these organisms.

Neither lysis nor protoplast release in suitable media could be observed. The significance of this result was discussed.

Examination of the lysozyme-treated cell suspensions in the phase contrast microscope revealed the cells to be normal in appearance.

The appearance of distorted cells in gram stained smears of the lysozyme-treated cells was observed with suspensions of Cl. hemolyticum L, Cl. butyricum L, Cl. multifermentans L, and Cl. lentoputrescens L.

Correlating these findings with the previous phase contrast microscope findings and the normal appearance of gram stained smears of the controls, it was suggested that the adverse effects of heat drying and fixing of the smears in the hypertonic solutions of sodium chloride, sodium thiocyanide,

and glycerol augmented the partial degradative effects of lysozyme on the cell envelopes.

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LITERATURE CITED

Abraham, E. P. 1939. Some properties of egg white lysozyme. Biochem. J. 33, 622-630.

Bachman, B. J. and D. M. Bonner. 1958. Protoplasts from Neurospora crassa. J. Bact. 78, 550-556.

Berger, L. R. and R. Weiser. 1957. The Beta glucosaminidase activity of egg white lysozyme. Biochem. et Biophys. Acta 26, 517-521.

Bramfit, W., A. C. Wardlaw, and J. T. Park. 1958. Development of lysozyme resistance and its association with an increased O-acetyl content of the cell wall. Nature 181, 1783-1784.

Eddy, A. A. 1958. The structure of yeast cell wall. II. Degradative studies with enzymes. Proc. Roy. Soc. (Lond.) B, 149, 425-440.

Epstein, L. A. and E. Chain. 1940. Some observations on the preparation and properties of the substrate of lysozyme. British J. Exp. Path. 21, 339-355.

Fleming, A. 1922. On a remarkable bacteriolytic element found in tissues and secretions. Proc. Roy. Soc. (Lond.) B, 93, 306-317.

Folin, O. and H. Malmros. 1929. An improved form of Folin's micro method for blood sugar determinations. J. Biol. Chem. 83, 115-120.

Frazer, D. and H. R. Mahler. 1957. The effects of Nucleas on the production of T_3 bacteriophage in protoplasts of Escherichia coli. Arch. Biochem. Biophys. 69, 166-177.

Ghysen, J. M. 1960. Acetylhexosamine compounds enzymically released from Micrococcus lysodeikticus cell walls. II. Enzymic sensitivity of purified acetylhexosamine-peptide complexes. Biochim. et Biophys. Acta 40, 473-480.

Ghysen, J. M. and M. R. J. Salton. 1960. Acetylhexosamine compounds enzymically released from Micrococcus lysodeikticus cell walls. I. Isolation and composition of acetylhexosamine and acetylhexosamine-peptide complexes. Biochim. et Biophys. Acta 40, 462-472.

Gooder, H. and W. R. Marxted. 1958. Protoplasts of Group A B hemolytic streptococci. Nature 182, 808-809.

Grula, E. A. and S. E. Hartsel. 1954. Lysozyme and morphological alterations induced in Micrococcus lysodeikticus. J. Bact. 68, 171-177.

Harris, J. O. 1961. Soil bacteria and corrosion. Proc. Sixth Ann. Appalachian Underground Corrosion Short Course 198-209.

Lederberg, J. and J. St. Clair. 1958. Protoplasts and L-type growth of Escherichia coli. J. Bact. 75, 143-157.

Mahler, H. R. and D. Frazer. 1956. Reproduction of bacteriophage T3 in protoplasts of Escherichia coli strain B. Biochim. et Biophys. Acta 22, 197-199.

McCarty, M. 1952. The lysis of Group A hemolytic streptococci by extracellular enzymes of Streptomyces albus. II. Nature of the substrate attacked by the enzyme. J. Expt. Med. 96, 569-580.

McQuillen, K. 1956. Capabilities of bacterial protoplasts. Symp. Soc. Gen. Microbiol. 6, 127-150.

_____. 1958. Lysis resulting from metabolic disturbances. J. Gen. Microbiol. 18, 498-512.

Meyer, F. L. and D. H. Northcote. 1958. A survey of the enzymes from the intestinal tract of Helix pomatia. J. Expt. Biol. 35, 639-648.

Meyer, K. and E. Hahnel. 1946. The estimation of lysozyme by a viscosometric method. J. Biol. Chem. 163, 723-732.

Meyer, K., E. Hahnel, and A. Steinberg. 1946. Lysozyme of plant origin. J. Biol. Chem. 163, 733-740.

Meyer, K., J. W. Palmer, T. Richard, and D. Khorazo. 1936a. The purification and properties of lysozyme. J. Biol. Chem. 113, 303-309.

Meyer, K., T. Richard, J. W. Palmer, and D. Khorazo. 1936b. The mechanism of lysozyme action. J. Biol. Chem. 113, 479-486.

Mitchell, P. and J. Moyle. 1956. Liberation and osmotic properties of the protoplasts of Micrococcus lysodiekticus. J. Gen. Microbiol. 15, 512-520.

Nakamura, O. 1923. Z. Immunforsch. 38, 425. Original not seen. Summary taken from Peterson and Hartsell (1955).

Northcote, D. H., K. J. Goulding, and R. W. Horne. 1958. Chemical composition and structure of cell wall of Chorella pyrenoidosa. Biochem. J. 70, 391-397.

Peterson, R. G. and S. E. Hartsel. 1955. The lysozyme spectrum of the gram negative bacteria. J. Infect. Dis. 96, 75-81.

Repaske, R. 1956. Lysis of gram negative bacteria by lysozyme. Biochim et Biophys. Acta 22, 189-190.

Repaske, R. 1958. Lysis of gram negative organisms and the role of versene. Biochim. et Biophys. Acta 30, 225-243.

Richmond, M. H. 1959a. Formation of a lytic enzyme by a strain of Bacillus subtilis. Biochim. et Biophys. Acta 33, 78-91.

_____. 1959b. Properties of a lytic enzyme produced by a strain of Bacillus subtilis. Biochim. et Biophys. Acta 33, 92-100.

Salton, M. R. J. 1952. Cell wall of Micrococcus lysodiekticus as the substrate of lysozyme. Nature 170, 746-747.

_____. 1953. Cell structure and the enzymic lysis of bacteria. J. Gen. Microbiol. 9, 512-523.

_____. 1955. Isolation of Streptomyces spp. capable of decomposing preparations of cell walls from various microorganisms and a comparison of their lytic activities with those of certain actinomycetes and myxobacteria. J. Gen. Microbiol. 12, 25-30.

_____. 1956. Studies of the bacterial cell wall. V. The action of lysozyme on cell walls of some lysozyme sensitive bacteria. Biochim. et Biophys. Acta 22, 495-506.

_____. 1957. The properties of lysozyme and its action of micro-organisms. Bact. Rev. 21, 82-99.

_____. 1959. An improved method for the detection of N-acetylamino sugars on paper chromatograms. Biochim. et Biophys. Acta 34, 308-312.

_____. 1960. Microbial cell walls. Ciba Lectures in Micro. Biochem. John Wiley & Sons, New York-London, 57-64 p.

Salton, M. R. J. and J. M. Ghysen. 1959. The structure of di- and tetrasaccharides released from the cell walls by lysozyme and the streptomyces F_1 enzyme and the β (1$\rightarrow$4) N-acetylhexosaminidase activity of these enzymes. Biochim. et Biophys. Acta 36, 552-554.

Sohler, A., A. H. Romano, and W. J. Nickerson. 1957. Biochemistry of the Actinomycetales. J. Bact. 75, 283-290.

Strange, R. E. and F. A. Dark. 1956. An unidentified amino sugar present in cell walls and spores of various bacteria. Nature 177, 186-188.

Warren, G. H., J. Gray, and R. Bartell. 1955. The lysis of Pseudomonas aeruginosa by lysozyme. J. Bact. 70, 614-619.

Weibul, C. 1953a. The isolation of protoplasts from Bacillus megaterium by controlled treatment with lysozyme. J. Bact. 66, 688-695.

Weibul, C. 1953b. The characterization of the protoplasmic constituents of Bacillus megaterium. J. Bact. 66, 696-701.

_____. 1956. Bacterial protoplasts; their formation and characteristics. Sixth Symp. Soc. Gen. Microbiol. 6, 111-125.

Wolff, L. K. 1927. Z. Immunitatsforsch. 50, 88. Original not seen. Summary taken from Meyer et al., 1936a.

LYSOZYME EFFECT ON SOME MEMBERS OF GENUS CLOSTRIDIUM

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From its first isolation (Fleming, 1922) to Salton and Ghysen's work in 1959, the interest in lysozyme has shifted from that of an applied research worker to that of a fundamental research worker. During this time lysozyme has been chemically characterized, its sources found, and its substrate in the bacterial cell wall determined. Its action on many organisms has been investigated by many workers with conflicting results.

This work was undertaken in an attempt to assess the degree of sensitivity of the clostridia to lysozyme. Several techniques were used for this purpose: turbidimetric, determination of release of reducing substances, and microscopic studies.

Seventeen Clostridium species and strains were used. The effect of lysozyme was observed on resting cell suspensions of the organisms. Armour Laboratories lysozyme was used throughout this work.

Optical density changes of the lysozyme treated resting cells were followed in the spectrophotometer at 600 mμ.

The amount of reducing sugar release by lysozyme treatment was determined, using the method of Folin and Malmros (1929).

Lysis or change in morphology was investigated with wet mounts in the phase contrast microscope and with gram stained smears in the light microscope.

The following results were obtained:

(A) All the organisms tested showed an ultimate significant increase in turbidity.

(B) Only Clostridium multifermentans L, Cl. tertium L, and Cl. lentoputrescens L yielded reducing material upon lysozyme treatment. These reducing values increased with the total population.

(C) Neither lysis nor protoplast release in suitable media could be observed. Phase contrast microscopy revealed no abnormality in cell morphology. Gram stained smears of lysozyme treated Cl. hemolyticum L, Cl. butyricum L, Cl. multifermentans L and Cl. lentoputrescens L showed distorted cells. The significance of these results is discussed.